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rbcL Transcript Levels in Tobacco Plastids Are Independent of Light: Reduced Dark Transcription Rate Is Compensated by Increased mRNA Stability

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The plastid *rbcL* gene, encoding the large subunit of ribulose-1,5-bisphosphate carboxylase, in higher plants is transcribed from a σ^{70} promoter by the eubacterial-type RNA polymerase. To identify regulatory elements outside of the *rbcL* −10/−35 promoter core, we constructed transplastomic tobacco plants with *uidA* reporter genes expressed from *rbcL* promoter derivatives. Promoter activity was characterized by measuring steady state levels of *uidA* mRNA on RNA gel blots and by measuring promoter strength in run-on transcription assays. We report here that the *rbcL* core promoter is sufficient to obtain wild-type rates of transcription. Furthermore, the rates of transcription were up to 10-fold higher in light-grown leaves than in dark-adapted plants. Although the rates of transcription were lower in the dark, *rbcL* mRNA accumulated to similar levels in light-grown and dark-adapted leaves. Accumulation of *uidA* mRNA from most *rbcL* promoter deletion derivatives directly reflected the relative rates of transcription: high in the light-grown and low in the dark-adapted leaves. However, *uidA* mRNA accumulated to high levels in a light-independent fashion as long as a segment encoding a stem–loop structure in the 5′ untranslated region was included in the promoter construct. This finding indicates that lower rates of *rbcL* transcription in the dark are compensated by increased mRNA stability.

INTRODUCTION

Plastid genes are transcribed by two RNA polymerases, one of which is related to the eubacterial α_2 ββ′ RNA polymerase (Igloi and Kössel, 1992; Gruissem and Tonkyn, 1993). The α_2 ββ′ homologs are encoded by the plastid genome; therefore, this RNA polymerase is referred to as the plastid-encoded plastid RNA polymerase (PEP). PEP promoter recognition is probably dependent on σ-like factors (Tiller et al., 1991; Tiller and Link, 1993a), which are encoded in the nucleus (Liu and Troxler, 1996; Allison, 1997; Isono et al., 1997a; Tanaka et al., 1997; Tozawa et al., 1998). PEP promoters are similar to the eubacterial σ^{70}-type promoters: core promoters comprise two conserved blocks of hexameric sequences (consensus; TTGACA and TATAAT) corresponding to the eubacterial −35 and −10 promoter elements. The hexamers are spaced 17 to 19 nucleotides apart; transcription initiates five to seven nucleotides downstream of the −10 box sequence. Accumulation of transcripts at a high level from poorly conserved PEP promoters depends on upstream activators such as the light-responsive promoter of the *psbD* gene encoding the D2 photosystem II subunit protein (Allison and Maliga, 1995; Kim and Mullet, 1995; To et al., 1996; Satoh et al., 1997). The second plastid RNA polymerase is nuclear encoded (NEP) and is likely to resemble a phage-type single-subunit enzyme related to the mitochondrial RNA polymerase (Lerbs-Mache, 1993; Hedtke et al., 1997). NEP promoters have a short, conserved sequence around the transcription initiation site (Hajdukiewicz et al., 1997; Kapoor et al., 1997; Hübschmann and Börner, 1998; Silhavy and Maliga, 1998a). PEP transcribes photosystem I and II genes, and NEP transcribes *accD* and the *rpoB* operon, whereas both polymerases transcribe the remaining genetic system and metabolic genes (reviewed in Maliga, 1998).

The plastid *rbcL* gene encodes the large subunit of ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39). The *rbcL* gene is transcribed by PEP from a single promoter, which has been mapped in a number of species, including tobacco (Shinozaki and Sugiura, 1982), maize, spinach, pea (Mullet et al., 1985), barley (Reinbothe et al., 1993), and Arabidopsis (Isono et al., 1997b). The tobacco *rbcL* gene has a PEP promoter with relatively well-conserved −35 and −10 elements (TTGCGC and TACAAT) and canonical spacing (18 nucleotides). In vitro studies have confirmed the importance of the −35 and −10 box spacing and sequence for *rbcL*

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promoter strength (Gruissem and Zurawski, 1985; Hanley-Bowdoin et al., 1985).

In vitro plastid transcription assays do not always reproduce the in vivo expression pattern (Wada et al., 1994). Therefore, we decided to test the role of sequences outside of the *rbcL* promoter core by expression of *uidA* reporter genes from *rbcL* promoter derivatives in vivo by using a transgenic approach (Svab and Maliga, 1993).

Sequences positioned between nucleotides −16 and −102 relative to the *rbcL* transcription initiation site are proposed to function in maize as a binding site for the chloroplast DNA binding factor 1 (CDF1) sequence-specific transcription factor. Segments of the CDF1 binding site are conserved between maize, pea, spinach, and tobacco (Lam et al., 1988). The region corresponding to the putative CDF1 binding site in tobacco is between nucleotides −16 and −96 with three conserved regions: promoter–distal CDF1 region I contains seven nucleotides of the maize GTATTTAG repeat; CDF1 region II corresponds to two A-rich sequences termed A boxes, whereas most of CDF1 region III is within the promoter core (underlined in Figure 1). We prepared a set of promoter deletion derivatives to test the role of the upstream elements in *rbcL* promoter function. DNA sequences downstream of the transcription initiation site were also tested for contribution to promoter activity. Although not yet confirmed for a higher plant, such *rbcL* promoter elements were found in the unicellular alga Chlamydomonas (Klein et al., 1994).

We report here that the *rbcL* core promoter is sufficient to obtain wild-type rates of transcription of the chimeric *uidA* genes. Therefore, no sequences outside of the promoter core significantly contribute to *rbcL* promoter function. Furthermore, the rates of *rbcL* transcription were significantly reduced in dark-adapted plants. Reduced rates of transcription resulted in lower steady state *uidA* mRNA levels in the dark, unless the 5′ segment of the *rbcL* 5′ untranslated region (5′ UTR) was included in the chimeric construct. This finding indicates that accumulation of *rbcL* mRNA in a light-independent manner is due to stabilization of the mRNA via its 5′ UTR compensating for reduced rates of transcription in the dark.

RESULTS

The *rbcL* Promoter Derivatives

A series of promoter deletion derivatives was constructed to test the role of sequences outside of the *rbcL* core for their

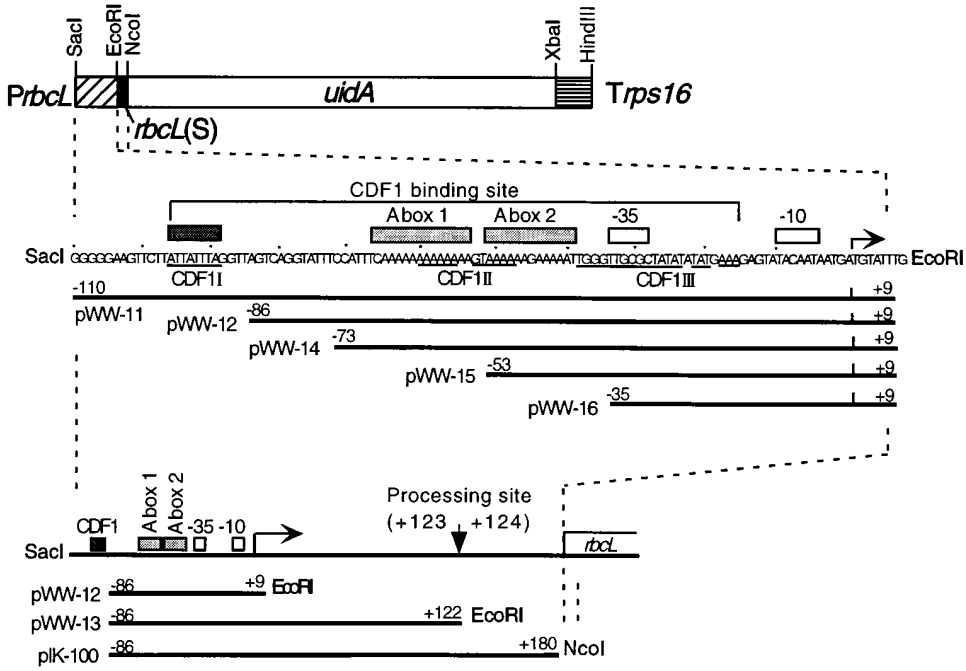

Figure 1. Chimeric *uidA* Genes to Test *rbcL* Promoter Activity.

P*rbcL* indicates the position of the *rbcL* promoter deletion derivatives; *uidA* encodes the β-glucuronidase reporter enzyme; T*rps16* refers to the 3′ UTR of the *rps16* ribosomal protein gene. The *rbcL* promoter sequence, with the transcription initiation site (horizontal arrow), the −10/−35 promoter elements, and conserved CDF1 regions (underlined) are shown below the chimeric gene. The *rbcL* promoter derivatives are identified by the pWW plasmid number.

contribution to promoter activity (Figure 1). The longest *rbcL* promoter in the 5′ deletion series includes CDF1 region I (−96/−89), the two A-rich A boxes (A box 1, −66/−54; A box 2, −51/−40), and nine nucleotides of the 5′ UTR in plasmid pWW11. In the shorter derivatives, one or more of these sequences have been systematically removed (pWW12, pWW14, pWW15, and pWW16; Figure 1).

Promoters in plasmids pWW12, pWW13, and pIK100 were designed to test for functional promoter elements downstream of the −35/−10 promoter core. These plasmids contain nine, 122, and 180 nucleotides downstream of the transcription initiation site, respectively (Figure 1). Note that the promoter driving *uidA* expression in plasmid pWW12 is shown in both the 5′ and the 3′ deletion series.

The *rbcL* promoter derivatives were cloned upstream of a *uidA* construct consisting of a ribosome binding site; *uidA*, encoding β-glucuronidase; and T*rps16*, the 3′ UTR of the *rps16* ribosomal protein gene to stabilize the mRNA. The chimeric *uidA* genes were cloned into the pPRV112A plastid transformation vector (Zoubenko et al., 1994) and introduced into the inverted repeat region of the tobacco plastid genome (Figure 2).

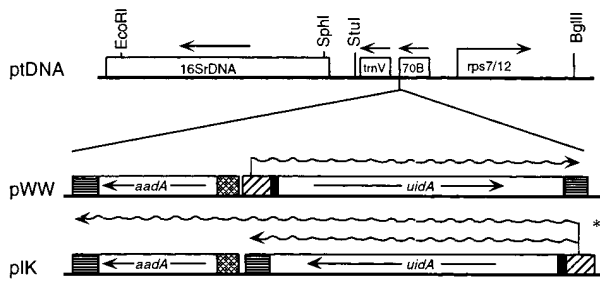

Figure 2. Map of the Tobacco Plastid Genome with the Selectable Marker *aadA* and Chimeric P*rbcL*::*uidA*::T*rps16* Gene.

The transgenes have been inserted into the *trnV–rps7/12* intergenic region. The 16S rDNA and *rps7/12* are plastid genes (Shinozaki et al., 1986). Wavy horizontal lines represent transcripts detected by the *uidA* probe in the pWW transgenic series and in the pIK100 transgenic plant. The asterisk marks the readthrough transcript. Arrows indicate the orientation of plastid genes. Hatched and stippled bars flanking *aadA* and *uidA* symbolize 5′ and 3′ regulatory regions.

Accumulation of mRNA to Wild-Type Levels in the Dark Depends on Sequences Downstream of the σ^{70}-Type Core Promoter

Steady state levels of the *rbcL* mRNA are similar in light-grown and dark-adapted tobacco plants (see below). Preliminary data indicated light-dependent accumulation of reporter mRNAs in some of the transgenic plants. Therefore, we studied mRNA accumulation in the leaves of dark-adapted plants (96 hr; DD plants) versus dark-adapted plants that had been illuminated for 24 hr (DL plants). Steady state mRNA levels for *rbcL* and the chimeric *uidA* genes are similar in DL plants and in plants grown in cycling light (16 hr of light and 8 hr of dark; data not shown).

In the transgenic plants, we probed for *rbcL* mRNA to examine mRNA accumulation from the native *rbcL* promoter and *uidA* mRNA to examine mRNA accumulation from the chimeric *rbcL* promoters. As a control, the 16S rRNA was used to detect RNA accumulation from the native rRNA operon promoter because accumulation of mRNA from the σ^{70}-type P*rrn-114* promoter is not dependent on light (Allison and Maliga, 1995). To control for equal loading, we probed the blots for the cytoplasmic 25S rRNA.

Accumulation of the *uidA* mRNA in the illuminated (DL) plants was comparable in all of the transgenic lines. Accumulation of *uidA* mRNA in the dark-adapted (DD) samples was lower than in the illuminated (DL) samples, unless the 5′ segment of the *rbcL* 5′ UTR between nucleotides +9 and +122 was incorporated in the chimeric promoters. The importance of this segment can be seen by comparing levels of mRNA in dark-adapted plants transformed with plasmids pWW12 and pWW13 (Figure 3).

There were two alternative explanations for high levels of *uidA* mRNA in the dark-adapted pWW13 and pIK100 plants. The pWW13 promoter fragment may have contained a promoter element downstream of the σ^{70} core that is responsible for maintaining high (illuminated) levels of transcription in the dark. Alternatively, the segment between positions +9 and +122 directed stabilization of the *uidA* mRNA in dark-adapted leaves.

A Segment of the *rbcL* 5′ UTR Protects the pWW13 *uidA* mRNA from Degradation

The two alternatives could be distinguished by measuring rates of transcription. Higher and lower rates of transcription correlating with higher and lower levels of steady state mRNAs in the dark-adapted plants would indicate a promoter element that enhances *rbcL* transcription in the dark. If, however, transcription rates from the chimeric promoters are higher in illuminated (DL) plants and lower in dark-adapted (DD) plants independent of the levels of mRNA, high steady state levels of *uidA* mRNAs in the pWW13 and pIK100 plants should be due to stabilization of the mRNAs in the dark.

In organello rates of transcription are determined by run-on transcription assays. First, chloroplasts are isolated while preserving plastid transcriptional elongation complexes. Transcriptional elongation is then continued in the presence of radioactive nucleotides. Radioactivity incorporated into specific mRNAs is determined by hybridization to immobilized DNA probes (Deng and Gruissem, 1995). The results of such a slot blot hybridization with the transgenic plants are

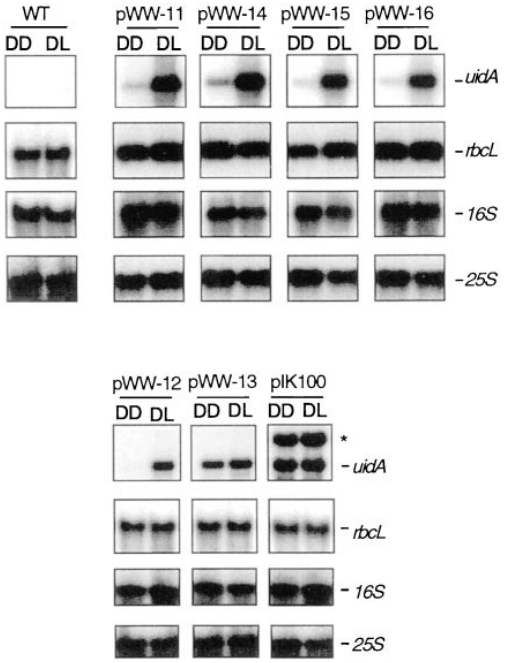

Figure 3. Accumulation of mRNAs from the Chimeric *uidA* Genes.

RNA samples were derived from dark-adapted (96 hr) plants (DD lanes) and dark-adapted (72 hr) and illuminated (24 hr) plants (DL lanes). The amount of total cellular RNA per lane was 3 μg for *rbcL* and *uidA* and 1 μg for the 16S rRNA. An asterisk marks a readthrough dicistronic (*uidA-aadA*) transcript in the pIK100 plastids. WT, wild type.

shown in Figure 4. Relative activities, normalized for the probe size, are given in Figure 5. The amount of incorporated UTP in plastid RNA in the dark-adapted (DD) plants was in the range of 0.33 to 1.49 pmol 5 $\times$ 10^6 plastids^{-1} 5 min^{-1}, whereas in the illuminated (DL) plants, it was in the range of 1.04 to 5.24 pmol 5 $\times$ 10^6 plastids^{-1} 5 min^{-1}. These values are similar to those found in 8-day-old barley seedlings (Figure 1 in Klein and Mullet, 1990).

The rate of *rbcL* transcription relative to the 16S rDNA is ~40% (Figure 5). This is also true of the chimeric *rbcL* promoters, indicating that all sequences relevant for transcription initiation from the *rbcL* promoter are present, even in the smallest fragment in the pWW16 construct. For both the native *rbcL* and the control 16S rDNA genes, the rates of transcription were relatively high in the illuminated (DL) sample and dropped dramatically (four- to 10-fold) in the dark-adapted (DD) sample. The pWW13 and pIK100 plants were no exception: transcription of the *uidA* gene was significantly reduced in the dark-adapted leaves, indicating that higher *uidA* mRNA levels in the dark are due to stabilization of the mRNA.

DISCUSSION

The *rbcL* σ^{70}-Type Promoter Core Is Sufficient for Normal Rates of Transcription in Vivo

Data presented in Figure 5 indicate that no sequence outside of the *rbcL* promoter core is necessary for full promoter activity. This conclusion is based on finding close to wild-type rates of *rbcL* transcription in all transgenic plants, including the pWW16 line, which carries the smallest (−35 to +9) *rbcL* promoter derivative, containing only a slightly larger fragment than the promoter core (−35 to −5).

It should be noted that the wild-type plants carry a single *rbcL* promoter per plastid genome, whereas the transgenic plants carry three: the wild-type *rbcL* promoter in the large single-copy region and two copies driving *uidA* expression in the inverted repeat region. Interestingly, introduction of two additional *rbcL* promoter copies does not effect *rbcL* promoter activity.

CDF1 binding in maize was more evident in bundle sheath chloroplasts than in mesophyll cell chloroplasts. Because CO_2 fixation by ribulose-1,5-bisphosphate carboxylase occurs in bundle sheath cells, CDF1 was proposed to be a cell type–specific regulator of *rbcL* expression (Lam et al., 1988).

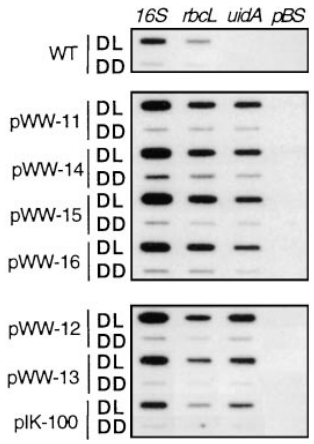

Figure 4. Slot Blot Analysis to Measure Rates of Plastid Gene Transcription.

RNA samples derived from dark-adapted (96 hr) plants (DD lanes) and dark-adapted (72 hr) and illuminated (24 hr) plants (DL lanes). Rates of transcription were measured for the 16S rRNA, *rbcL*, and chimeric *uidA* genes expressed from the *rbcL* promoter derivatives. Plasmid pBluescript II KS+ (pBS) DNA was used as a control for nonspecific hybridization of the run-on transcripts. The nonlabeled DNA (0.5 pmol per slot) was probed with ^{32}P-labeled RNA from the lysed transcription assay of 5 $\times$ 10^6 chloroplasts. Data shown on this representative blot may be slightly different from the averages of multiple experiments shown in Figure 5. WT, wild type.

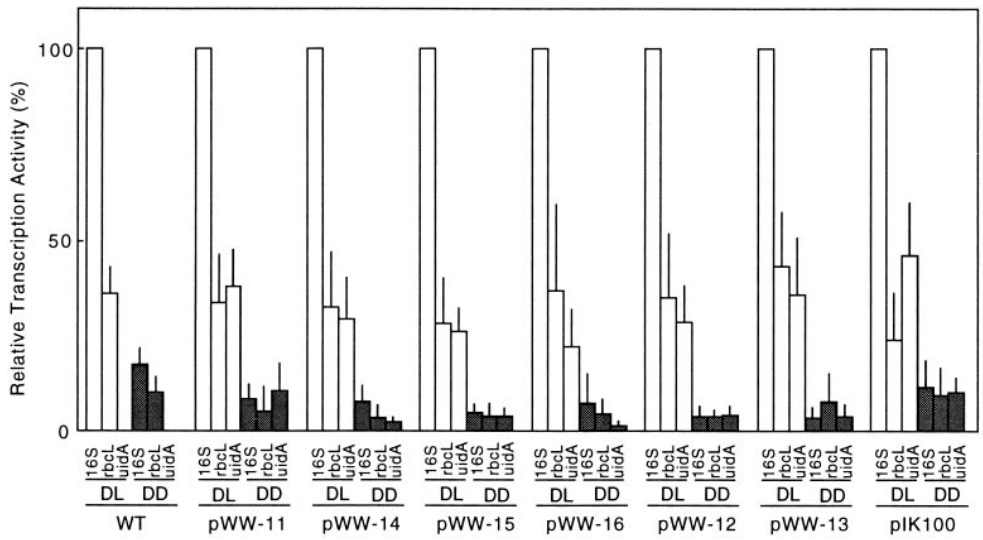

Figure 5. Relative Rates of Transcription of the 16S rDNA, *rbcL*, and Chimeric *uidA* Genes.

Gene-specific activities were calculated as UTP incorporated (picomoles) per 10^6 plastids per 1-kb length of DNA probe during a 5-min assay. The values are given as the percentage of the 16S rDNA transcription rate in the light (100%) in arbitrary units (mean ±SD). Under the experimental conditions used here, the actual 16S rDNA transcription rate may be underestimated, resulting in overestimation of the relative *rbcL* transcription rates. The values were calculated from four to six independent slot blot hybridization experiments, except for the wild-type plants, for which the numbers were calculated from two experiments. Abbreviations are as given in the legend to Figure 4.

This still may be the case. Given the limited sequence conservation between maize and tobacco CDF1 binding sites, and because tobacco is a C_3 rather than a C_4 plant, the lack of a role for the CDF1 chloroplast DNA binding factor in tobacco does not necessarily exclude a role for CDF1 sequences in C_4 plastids.

In *Escherichia coli*, the AT-rich UP element is localized between nucleotides −40 to −60 upstream of the transcription initiation site and stimulates transcription by a factor of 30 in vivo (Ross et al., 1993). Tobacco *rbcL* CDF1 region II (A boxes) is similarly spaced relative to the *rbcL* promoter core. Sequences upstream of the −35 box in the *psbD* promoter contain binding sites for a factor regulating *psbD* transcription (Allison and Maliga, 1995; Kim and Mullet, 1995; Christopher, 1996; To et al., 1996; Satoh et al., 1997). However, the two A box regions upstream of the tobacco *rbcL* promoter do not seem to play a role in regulating transcription. This is also true of sequences downstream of the promoter core that are required for full *rbcL* promoter strength in Chlamydomonas (Klein et al., 1994).

Transcription from σ^{70}-Type Promoters Is Light Regulated in Chloroplasts

We have shown that steady state levels of *rbcL* mRNA in tobacco are comparable in dark-adapted (DD) and light-induced (DL) leaves. Also, *uidA* mRNA accumulates to similar levels in dark-adapted (DD) and light-induced (DL) leaves from the P*rrn-114* promoter, which drives rRNA transcription in leaves (Allison and Maliga, 1995; Vera and Sugiura, 1995; Allison et al., 1996). Given light-independent accumulation of mRNAs from these σ^{70}-type PEP promoters, we had expected to find that transcription from these promoters is constitutive. Interestingly, this is not the case. Transcription rates in dark-adapted leaves are four to 10 times lower than in the illuminated leaves for both *rbcL* and the 16S rDNA. Enhanced rates of plastid transcription by light have been reported in several species, including spinach (Deng and Gruissem, 1987), barley, maize (Baumgartner et al., 1993), sorghum (Schrubar et al., 1990), wheat (Kawaguchi et al., 1992), pea (DuBell and Mullet, 1995), and Arabidopsis (Hoffer and Christopher, 1997). Responsiveness of plastid gene transcription to light also depends on the developmental stage of the plastid (Klein and Mullet, 1990; Baumgartner et al., 1993).

Multiple mechanisms may lead to light-induced PEP activity. Light-induced synthesis of the components of the PEP transcription machinery may be one such mechanism. Candidates for this type of regulation are the PEP σ factor genes, whose transcription is induced by light (Liu and Troxler, 1996; Isono et al., 1997a; Tanaka et al., 1997; Tozawa et al., 1998). Conversion of the eubacterial-type PEP core (B form) into the more complex A form during light-induced development

may also contribute to light-induced PEP activity (Pfannschmidt and Link, 1994, 1997). An additional mechanism could be reversible phosphorylation of the σ factors shown to regulate rates of transcription in vitro (Tiller and Link, 1993b) and in vivo (Christopher et al., 1997). The in vivo role of phosphorylation in regulating plastid gene transcription seems to be corroborated by the purification of a PEP-associated protein kinase, which phosphorylates chloroplast σ-like factors in vitro (Baginsky et al., 1997).

The 5′ UTR Is Responsible for Stabilizing the *rbcL* mRNA in the Dark

The experimental observations of light-dependent *rbcL* transcription and similar steady state levels of *rbcL* mRNA in dark-adapted (DD) and illuminated (DL) leaves suggest stabilization of mRNA as a compensating mechanism for the lower rates of transcription in the dark. The in vivo study of *uidA* expression from *rbcL* promoter derivatives points to the 5′ UTR as the source of the stabilizing sequences. In the plastids of higher plants, studies of mRNA stability thus far have been limited to the role of the 3′ UTR. The 3′ UTR frequently contains a short stem–loop structure associated with a high molecular weight complex. This complex contains a 3′ to 5′ exonuclease (PNPase, or 100-kD polynucleotide phosphorylase), two endoribonucleases, and at least four additional proteins involved in 3′ end formation and degradation of the mRNA (Stern and Gruissem, 1987; Hayes et al., 1996; Yang et al., 1996). A putative short stem–loop structure is also present in the *rbcL* 5′ UTR. This is disrupted by the deletion in the pWW12 promoter derivative but is present in the pWW13 and pIK100 promoters (Figure 6). The segment of the *rbcL* 5′ UTR containing this stem–loop structure is highly conserved during evolution, suggesting function in vivo (Figure 6).

The tobacco *rbcL* mRNA has an RNA processing site in the 5′ UTR between nucleotide positions +123 and +124, which are marked in Figures 1 and 6 (Hanley-Bowdoin et al., 1985; Mullet et al., 1985; Reinbothe et al., 1993). This RNA processing site is absent in all *rbcL* promoter derivatives except pIK100 (Figure 1). The chimeric mRNA is unstable in dark-adapted (DD) pWW12 plants, whereas it is stabilized in pWW13 plants (Figure 3). Both sets of transgenic plants lack the RNA processing site. Therefore, stabilization of chimeric mRNAs is independent of RNA processing at this site.

We report here regulation of plastid mRNA stability via the mRNA 5′ UTR. To our knowledge, no such information has been published for a higher plant. However, there are several well-documented cases for regulation of mRNA stability via the 5′ UTR in the unicellular alga Chlamydomonas. The examples include studies of nuclear mutations that target the 5′ UTR and thereby destabilize the *psbD* mRNA (reviewed in Mayfield et al., 1995; Rochaix, 1995). A transgenic approach was used to confirm the role of the *rbcL* (Salvador et al., 1993) and *petD* (Sakamoto et al., 1993, 1994) 5′ UTRs and of the *atpB* mRNA

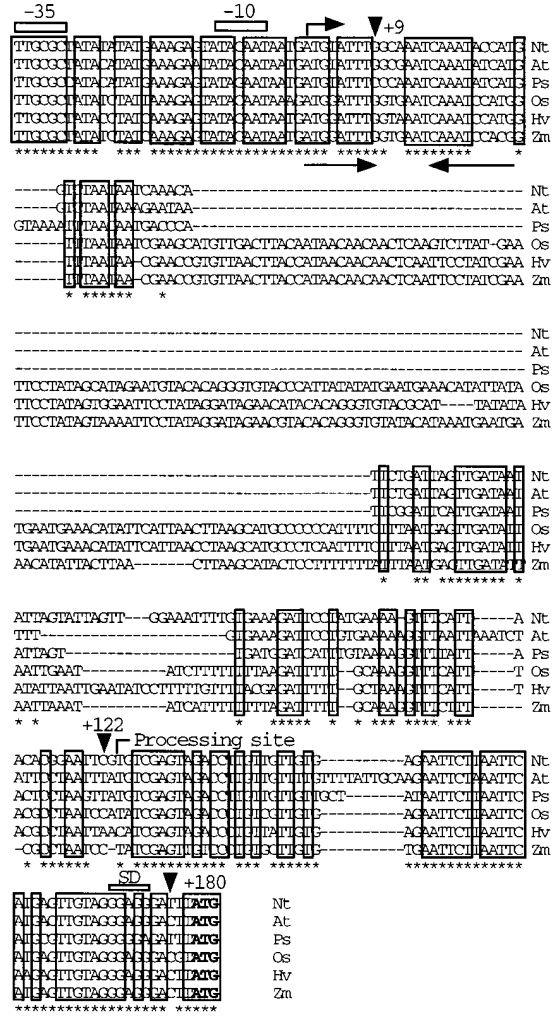

Figure 6. DNA Sequence Alignment of Regions Containing the *rbcL* 5′ UTR in Higher Plants.

Nt, tobacco (Shinozaki and Sugiura, 1982); At, Arabidopsis (Isono et al., 1997b); Ps, pea (Zurawski et al., 1986); Os, rice (Silhavy and Maliga, 1998b); Hv, barley (Reinbothe et al., 1993); and Zm, maize (Mullet et al., 1985). Nucleotides conserved in all six species are boxed; those conserved in five of the six species are marked by asterisks under the alignment. The *rbcL* translation initiation codon (ATG) is shown in boldface. Positions of the −10 and −35 promoter elements, the Shine-Dalgarno (SD) sequence, the processing site, and the 3′ end of promoter derivatives (filled triangles) are marked above the sequence; inverted repeats in tobacco are marked by horizontal arrows below the sequence. The size of the repeated region is slightly different in the other species. Dashes were introduced to optimize alignment.

3′ UTR (Stern et al., 1991; Stern and Kindle, 1993) in mRNA maturation and turnover. The role of the short stem–loop structure, and possibly of other RNA *cis* sequences, in stabilizing the higher plant *rbcL* mRNAs in the dark remains to be determined.

METHODS

Plasmid Construction

A series of 5′ deletions of the upstream sequence of the *rbcL* transcription initiation site was amplified using pIK36 containing the *rbcL* promoter as a template. Plasmid pIK36 is a pBluescript KS+ (Stratagene, La Jolla, CA) vector derivative carrying an SpeI-SacII plastid DNA fragment spanning nucleotides 56,389 to 57,750 in the plastid genome (Shinozaki et al., 1986). The *rbcL* promoter derivatives were amplified as SacI (at the 5′) and EcoRI (at the 3′) fragments with the following primers: pWW11, 5′-GGGAGCTCGGGGGAAGTTCTTATTA-3′ (5′ end point −110) and 5′-GGAATTCAAATACATCATTATTG-3′ (3′ end point at +9); pWW12, 5′-GGGAGCTCGTCGACTAGTCAGGTATTTC-3′ (5′ end point −86) and 5′-GGAATTCAAATACATCATTATTG-3′ (3′ end point at +9); pWW14, 5′-GGGAGCTCCCATTTCAAAAAAAAAA-3′ (5′ end point −73) and 5′-GGAATTCAAATACATCATTATTG-3′ (3′ end point at +9); pWW15, 5′-GGGAGCTCGTAAAAAAGAAAAATTGGG-3′ (5′ end point −53) and 5′-GGAATTCAAATACATCATTATTG-3′ (3′ end point at +9); pWW16, 5′-GGGAGCTCGTTGCGCTATATATATG-3′ (5′ end point −35) and 5′-GGAATTCAAATACATCATTATTG-3′ (3′ end point at +9); pWW13, 5′-GGGAGCTCGTCGACTAGTCAGGTATTTC-3′ (5′ end point −86) and 5′-CGAATTCCGTGTTAATGA-3′ (3′ end point at +122). The fragments obtained by amplification with these primers were digested with EcoRI and SacI and ligated into SacI-EcoRI–digested plasmid pLAA25A (Zoubenko et al., 1994) to obtain plasmids pWW12, pWW13, pWW14, pWW15, and pWW16. Plasmid pLAA25A is a pRRV112A plastid transformation vector derivative with a selectable spectinomycin resistance (*aadA*) gene and a promoterless *uidA* coding region with (1) a synthetic ribosome binding site and (2) the *rps16* ribosomal protein gene 3′ untranslated region (UTR; T*rps16*; Zoubenko et al., 1994). Cloning of *rbcL* promoter fragments upstream of the ribosome binding site created the pWW series of P*rbcL*::*rbcL*(S)::*uidA*::T*rps16* reporter genes.

Plasmid pIK100 is a pPRV111B plastid transformation vector derivative (Zoubenko et al., 1994) in which the *uidA* reporter gene (P*rbcL*::*uidA*::T*rbcL*) is cloned as a SalI-HindIII fragment in the polycloning site in the same orientation as the selectable marker spectinomycin resistance (*aadA*) gene. P*rbcL* in plasmid pIK100 is contained in a SalI (at the 5′ end) and NcoI (3′) fragment, including nucleotides between 57,319 and 57,586 of the plastid genome (Shinozaki et al., 1986). This region includes sequences −86/+181 relative to the transcription initiation site. The SalI and NcoI sites were created by site-directed mutagenesis using oligonucleotides 5′-GGAAATACCTGACTAGTCGACCTAAATAATAAGAACTTC-3′ and 5′-GTCTCTGTTTGTGGTGCCATGGATCCCTCCCTACAAC-3′, respectively. The *uidA* coding segment is identical in the pWW plasmids and in plasmid pIK100, and it is contained in an NcoI-XbaI fragment. T*rbcL* (XbaI-HindIII fragment) has been described previously (Staub and Maliga, 1994). The chimeric P*rbcL*:: *uidA*::T*rbcL* gene was cloned in plastid vector pPRV111B (Zoubenko et al., 1994) to yield plasmid pIK100. Plants transformed with plasmid pIK100 were made available by I. Kanevski (Waksman Institute).

Plastid Transformation

Tobacco (*Nicotiana tabacum* cv Petit Havana) plants were grown aseptically on RM medium containing agar-solidified Murashige and Skoog salts (Murashige and Skoog, 1962) and sucrose (3%). Tungsten particles (1 μm) coated with plasmid DNA were introduced into the leaves by using the DuPont PDS1000He Biolistic gun at 1100 psi (Svab and Maliga, 1993). Transgenic shoots were selected on RMOP medium containing 500 mg/L spectinomycin dihydrochloride (Svab et al., 1990). Homoplastomic transformed lines were identified by DNA gel blot analysis after two cycles of plant regeneration on the same selective spectinomycin medium. Transgenic cuttings were rooted on Murashige and Skoog agar medium (Murashige and Skoog, 1962).

Light Treatment

Transgenic plants were rooted aseptically on solid RM media in culture boxes and maintained under a 16-hr-light and 8-hr-dark cycle for 4 to 8 weeks. For dark adaptation, plants were placed in complete darkness for 3 days. Subsequently, plants were either maintained in the dark or returned to white light (cool-white fluorescent bulbs at ~10,000 lux) for 24 hr.

RNA Gel Blot Analysis

Total cellular RNA was isolated from fully expanded leaves frozen in liquid nitrogen by using TRIzol (Gibco BRL), following the manufacturer's protocol. The RNA was electrophoresed on 1% agarose–formaldehyde gels and transferred to a Hybond N membrane (Amersham) by using the PosiBlot transfer apparatus (Stratagene). Blots were hybridized using the rapid hybridization buffer (Amersham) at 65°C, with ^{32}P-labeled double-stranded DNA probes generated by random primed labeling (Boehringer Mannheim).

Chloroplast Isolation and Run-on Transcription

The plants were grown aseptically on RM medium containing agar-solidified Murashige and Skoog salts and 3% sucrose in Magenta boxes. Chloroplast run-on transcription assays were performed according to Deng and Gruissem (1995). Briefly, leaves (5 to 10 g) of aseptically grown plants were homogenized in an isolation buffer (25 mM Hepes-KOH, pH 7.6, 350 mM sorbitol, and 2 mM EDTA) in a Waring blender. Intact plastids were isolated by centrifugation (10,000*g*) on a 10 to 80% Percoll gradient. The chloroplast number was determined by counting in a hemocytometer. Run-on transcription was performed using α-^{32}P-UTP (specific activity of 400 mCi/mmol) and 10^7 chloroplasts in the presence of 20 mg/mL heparin. After an 8-min incubation at 25°C, duplicate aliquots were spotted on DE-81 paper (Whatman, Clifton, NJ) for measurement of total UTP incorporation into RNA. Radioactive run-on transcripts were purified by phenol extraction and hybridized with nonradiolabeled 16S rDNA, *rbcL*, and *uidA* gene probes and pBS plasmid DNA (0.5 pmol each) slot blotted on nylon membranes. The probes were the 16S rDNA 1.28-kb EcoRI-SphI fragment (Shinozaki et al., 1986); *rbcL* 1.3-kb EcoRI-XbaI fragment (Shinozaki et al., 1986); *uidA* 1.87-kb NcoI-XbaI

fragment (plasmid pLAA25A; Zoubenko et al., 1994); and plasmid pBluescript II KS+ EcoRI-digested plasmid DNA (Stratagene). Slot blots were probed with lysed assays of 5×10^6 chloroplasts. Radioactivity hybridizing with the probes was determined by measuring radioactivity in excised bands by using a liquid scintillation counter.

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